

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



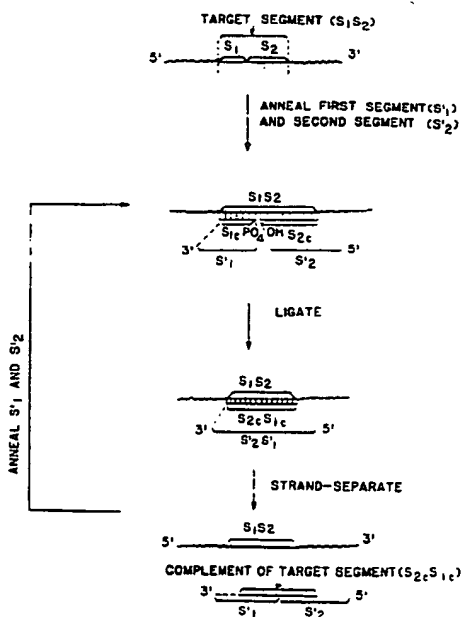
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁴ : C12Q 1/68, C12P 19/34 C12N 15/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 89/09835 (43) International Publication Date: 19 October 1989 (19.10.89)</p>
<p>(21) International Application Number: PCT/US89/01471 (22) International Filing Date: 7 April 1989 (07.04.89) (30) Priority data: 179,478 8 April 1988 (08.04.88) US (71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US). (72) Inventor: ORGEL, Leslie, E. ; 6102 Terryhill Drive, La Jolla, CA 92037 (US). (74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published With international search report.</p>

(54) Title: LIGASE-BASED AMPLIFICATION METHOD

(57) Abstract

The invention provides methods for amplification of a nucleic acid segment of interest wherein a DNA ligase catalyzes ligation of DNA segments hybridized to the segment of interest or the segment with the sequence complementary to that of the segment of interest. The methods of the invention have broad application to molecular biology and practical applications thereof, in which a particular nucleic acid segment needs to be detected or isolated. Among the applications of the methods of the invention is amplifying nucleic acid analytes for permitting or enhancing their detectability in nucleic acid probe hybridization assays.



-16-

to adjoining subsegments of the complement of the target segment and are ligated thereon.

Other aspects of the invention, such as, e.g., kits for carrying out the aforementioned processes, will be apparent to the skilled in the art from the present disclosure. All such kits would comprise, in a first container, a DNA ligase, and, in a second container, at least one pair of DNA segments to provide amplification of a target segment in a sample of nucleic acids in accordance with the invention. In kits to be used to amplify a target segment (or complement thereof) in accordance with the invention approximately exponentially, with the number of cycles of annealing of segment to target segment, ligation of the annealed segments, and strand-separation of newly ligated segments from template on which ligation occurred, the second container of a kit would have at least two pairs of DNA segments, one pair to be ligated upon annealing to target segment and the other to be ligated upon annealing to complement of target segment. In kits according to the invention for detection of a nucleic acid analyte, which comprises a target segment of known sequence, after amplification of the target segment (or complement thereof) in accordance with the invention, there may be, in addition, a third container which comprises a nucleic acid probe that is labeled for detection and that has a sequence of at least about 8, and more preferably at least about 20, nucleotides whereby the probe can hybridize to a segment with the sequence of target segment, or complement thereof, that has been amplified in the amplification process. Alternatively, in kits of the invention for such detection, at least one of the nucleic acid segments to be ligated in the amplification process (and held in said second container) may be labeled for detection in a way that does not interfere

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	LI	Liechtenstein	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

-1-

LIGASE-BASED AMPLIFICATION METHOD

TECHNICAL FIELD

The present invention concerns molecular biology
5 and nucleic acid chemistry.

More particularly, the invention relates to
amplification of nucleic acid segments of interest.

Among the applications of the present invention
is amplifying nucleic acid analytes for permitting or
10 enhancing their detectability in nucleic acid probe
hybridization assays.

BACKGROUND OF THE INVENTION

A persistent problem in molecular biology,
15 including applications thereof such as engineering
microorganisms to make a protein of interest or detecting
a disease-causing microorganism or a defective gene by a
nucleic acid probe hybridization assay of an appropriate
sample, is that a nucleic acid of interest, if present at
20 all in a sample, is present at very low copy number and
as a very small mole fraction of total nucleic acid. The
problem is further exacerbated in many cases by the
presence, in a sample, of nucleic acids that are close in
sequence to the one of interest. Thus, isolating from a
25 sample, or even simply detecting the presence in a
sample, of a particular nucleic acid of interest often
requires methods that are highly sensitive and highly
specific.

One approach to this need for high sensitivity
30 and specificity is to treat a sample to "amplify" a
nucleic acid of interest, or a segment thereof that is
pertinent to the problem at hand, before attempting to
isolate or detect same. "Amplifying" a nucleic acid or
nucleic acid segment in a sample means treating the
35 sample to increase both the copy number of the nucleic

-2-

acid or segment of interest and the ratio of that copy number to the sum of the copy numbers of all nucleic acids or nucleic acid segments with the same number of bases as the nucleic acid or segment of interest. Thus, amplification requires both a means to replicate the nucleic acid or segment of interest and a means to replicate such nucleic acid or segment preferentially to (i.e., at a faster rate than) others in the sample.

Numerous methods of amplification of a particular nucleic acid of interest are known to those skilled in molecular biology. Until recently all these methods were in vivo methods, which rely on manipulations of cultures of microorganisms to cause more rapid growth of microorganisms that harbor the nucleic acid of interest than of those which do not or cause more rapid transcription or replication of chromosomal DNA segments, or episomal or viral nucleic acids, that include a nucleic acid segment of interest, than of other nucleic acids present in the microorganisms.

Recently, an in vitro method, known as the "PCR", or "polymerase chain reaction", method for amplifying a particular nucleic acid segment of at least partially known sequence in a complex mixture of nucleic acids has been devised. See Saiki et al., Science 230, 1350-1354 (1985); Mullis, U.S. Patent No. 4,683,202; Saiki and Erlich, U.S. Patent No. 4,683,194; and Mullis et al., U.S. Patent No. 4,683,195. This method has broad application to molecular biology and practical applications thereof, in which a particular nucleic acid segment needs to be detected or isolated. A major application of the method is with nucleic acid probe hybridization assays for detection of "target" nucleic acid segment analytes, that occur at low copy number in specimens of body fluids or the like taken for testing for the presence of, e.g., a pathogenic virus or

-3-

microorganism or a defective gene. The PCR method employs a DNA polymerase to catalyze formation, from the 3'-OH of a primer oligonucleotide which hybridizes to a 3'-subsegment of a template, of a DNA segment complementary in sequence to that of the template. By employing two primer oligonucleotides, one complementary in sequence to a 3'-subsegment of a target segment and the other identical in sequence to a 5'-subsegment of the target segment, and carrying out DNA polymerase catalyzed chain extensions repeatedly from the primers, with a strand-separation step and primer-reannealing step between each chain-extension, a nearly exponential (as a function of number of repeats) increase in copy number of target segment and an at most linear (as a function of number of repeats) increase in copy number of other segments (to which a primer might hybridize) are, in principle, achieved. In the PCR method, replication of the segment of interest is provided by DNA polymerase-catalyzed chain-extension and such replication preferentially of target segment and complement of target segment (i.e., the segment with the sequence complementary to that of target segment) is provided by the requirement of a DNA polymerase for a primer stably hybridized to a nucleic acid template to initiate catalysis of chain-extension.

The present invention employs DNA ligation, in place of primer-initiated DNA chain-extension, to effect amplification of a target nucleic acid segment of known sequence.

The present invention relies on the properties of DNA ligases, to join a first DNA segment, that is hybridized to a subsegment of a template and has a 5'-phosphate, to a second DNA segment, that is hybridized to an adjoining subsegment of the template and has a 3'-hydroxyl immediately adjacent the 5'-phosphate of the first DNA segment.

-4-

Many DNA ligases are available. Every organism more advanced than a virus requires a DNA ligase for viability, and even many viruses have a gene to encode such a ligase. The two most highly characterized DNA
5 ligases are that which is the product of the lig gene of Escherichia coli (called "E. coli DNA ligase") and that which is the product of gene 30 of bacteriophage T4 (called "T4 DNA ligase"). See Engler and Richardson, "DNA Ligases," in The Enzymes, Vol. 15B, P.D. Boyer, ed.,
10 Academic Press, New York, New York, 1982, pp. 3-30; Olivera, Meth. Enzymol. 21, 311 (1971); Weiss, Meth. Enzymol. 21, 319 (1971); and Moderich and Lehman, J. Biol. Chem. 245, 3626 (1970). Both T4 DNA ligase and E.coli DNA ligase are available commercially. E.coli DNA
15 ligase is particularly well suited for catalyzing ligations in accordance with the methods of the present invention.

Prior to the present invention, it was not appreciated in the art that methods for amplification could be based on ligation catalyzed by a nucleic
20 acid ligase.

SUMMARY OF THE INVENTION

It has been discovered that amplification of a
25 nucleic acid segment of interest, which has a known sequence, can be accomplished utilizing a DNA ligase to catalyze ligation of suitable DNA segments hybridized appropriately to the segment of interest or the complement of the segment of interest.

30 In accordance with the invention, the replication of the segment of interest required for amplification is provided by DNA ligase-catalyzed ligation of a 5'-phosphate-terminated, single-stranded DNA segment to an immediately adjacent, 3'-hydroxyl-
35 terminated, single-stranded DNA segment and the

-5-

preferential aspect of the replication, also required for amplification, is provided by the requirement, for DNA ligase-catalyzed ligation of single-stranded DNA segments, that the segments to be ligated be stably
5 hybridized to adjoining subsegments of a DNA template, with the nucleotide with the 5'-terminal phosphate of one of the segments to be ligated and the nucleotide with the 3'-hydroxyl of the other of the segments to be ligated base-paired to adjoining nucleotides of the template.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates an embodiment of the invention wherein an increase in copy number of a DNA, with a segment, which is denominated "complement of
15 target segment" in the Figures and has the sequence complementary to that of the target segment, occurs linearly (with the number of ligation reaction steps).

Figure 2 schematically illustrates an embodiment of the invention wherein increases in copy numbers, of
20 both a DNA, which has a segment with the sequence of the target segment, and a DNA, which has a segment with the sequence complementary to that of the target segment, occur exponentially (with the number of ligation reaction steps).

Figure 3 schematically illustrates an embodiment of the invention wherein increases in copy numbers, of
25 both a DNA, which has a segment with the sequence of the target segment, and a DNA, which has a segment with the sequence complementary to that of the target segment, occur exponentially (with the number of ligation reaction
30 steps) and wherein target segment and its complement occur together in a double-stranded nucleic acid in the sample to which the method of the invention is applied.

In the Figures, a phosphate group is shown at
35 the 5'-end of an annealed first segment (S_1') and an

-6-

hydroxyl group is shown at the 3'-end of an annealed second segment (S_2') to be ligated to the annealed first segment. Similarly, a phosphate group is shown at the 5'-end of an annealed fourth segment (S_2'') and an
5 hydroxyl group is shown at the 3'-end of an annealed third segment (S_1'') to be ligated to the annealed fourth segment.

In the Figures, $S_2'S_1'$ represents the single-stranded DNA made by the ligation of S_1' to
10 S_2' and $S_1''S_2''$ represents the single-stranded DNA made by the ligation of S_1'' to S_2'' .

As indicated schematically in the Figures:
 S_1' consists of a 5'-subsegment (S_{1C}), which has the same number of bases as and is complementary in sequence
15 to the 5'-subsegment (S_1) of target segment, and, optionally, a 3'-subsegment, which is indicated in the Figures by dashes. S_2' consists of a 3'-subsegment (S_{2C}), which has the same number of bases as and is complementary in sequence to the 3'-subsegment (S_2) of
20 target segment, and, optionally, a 5'-subsegment, which is indicated in the Figures by dots. S_1'' consists of a 3'-subsegment (S_1), which has the same number of bases and the same sequence as the 5'-subsegment (S_1) of target segment, and, optionally, a 5'-subsegment, which
25 is indicated in the Figures by wavy dashes (i.e., ~). S_2'' consists of a 5'-subsegment (S_2), which has the same number of bases and the same sequence as the 3'-subsegment (S_2) of target segment, and, optionally, a 3'-subsegment, which is indicated in the Figures by
30 open circles. A target segment (S_1S_2) consists of a 5'-subsegment (S_1) and a 3'-subsegment (S_2) and may be only a part of a longer strand of nucleic acid. The complement of target segment (i.e., the segment with the same number of bases as and the sequence complementary to
35 that of target segment) ($S_{2C}S_{1C}$) consists of a

-7-

5'-subsegment (S_{2C}), which has the same number of bases as and the sequence complementary to that of the 3'-subsegment of target segment, and a 3'-subsegment (S_{1C}), which has the same number of bases as and the sequence complementary to that of the 5'-subsegment of target segment. Like target segment, the complement thereof may also be only part of a longer strand of nucleic acid.

The 3'-subsegment of S_1' , indicated in the Figures by dashes; the 5'-subsegment of S_2' , indicated in the Figures by dots; the 5'-subsegment of S_1'' , indicated in the Figures by wavy dashes; and the 3'-subsegment of S_2'' ; indicated in the Figures by open circles, if present in S_1' , S_2' , S_1'' or S_2'' , respectively, do not contribute significantly to the stability of the hybrids, of S_1' and S_2' with target segment (or with S_1'' ligated to S_2'') and S_1'' and S_2'' with complement of target segment (or with S_1' ligated to S_2') required for the ligations of S_1' to S_2' and S_1'' to S_2'' catalyzed by a DNA ligase.

DETAILED DESCRIPTION OF THE INVENTION

In one of its aspects, the present invention entails a process for amplifying a DNA segment with the sequence complementary to that of a target nucleic acid segment of known sequence, which process comprises:

- (1) annealing to said target segment (A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment of said target segment, and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment of said target segment, provided

-8-

that the sequence of the segment, which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment is complementary to the sequence of said target segment;

5 (2) ligating said annealed first and second DNA segments;

 (3) prior to repeating step (1), strand-separating said target segment from said ligated first and second DNA segments; and

10 (4) repeating at least steps (1) and (2) at least once.

 This process of the invention, which entails repeating, as often as desired or, for the purpose for which the amplification is carried out, necessary, cycles
15 of annealing, to adjoining subsegments of a target nucleic acid segment of known sequence, of two DNA segments (one for each of the adjoining subsegments of the target), ligation of the annealed segments, and strand-separation of the ligated segments from the target
20 segment, results in an approximately linear increase in copy number of the ligated segments (and, therefore, copy number of complement of target segment) as a function of the number of such cycles.

 The process can be carried out first, for a
25 number of cycles, with two segments (e.g., S_1' and S_2') that hybridize to the target segment and then, for an additional number of cycles, with two different segments, which hybridize to adjoining subsegments of the segment, $S_2'S_1'$, made upon ligation of the two
30 segments employed in the initial number of cycles. For the second set of cycles, some subsegment of the segment, $S_2'S_1'$, made upon ligation of the two segments employed in the initial number of cycles is, in effect, a second target segment. This second target segment is
35 preferably, but not necessarily, the complement of the

-10-

Reference herein to a "5'-terminal" moiety (e.g., a phosphate group) mean a moiety that is covalently joined to the 5'-carbon of the 5'-terminal nucleotide of a nucleic acid strand. Reference herein to
5 a "3'-terminal" moiety (e.g., an hydroxyl group) means a moiety that is covalently joined to the 3'-carbon of the 3'-terminal nucleotide of a nucleic acid strand.

Note that a "target segment", or "complement of target segment" is defined by sequence and number of
10 bases. A target segment (or complement thereof) in a sample may exist as part of a one or more longer strand(s) of nucleic acid or freely as a separate strand or in both states. The amplification processes of the invention effect increases in copy numbers in a sample of
15 complement of target segment or both complement of target segment and target segment.

In another of its aspects, the invention entails a process for amplifying a target nucleic acid segment of known sequence or the complement of said target segment
20 which process comprises:

(1) annealing to said target segment (A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary
25 to that of a 5'-subsegment of said target segment and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment of said target segment, provided
30 that the sequence of a segment which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment is complementary to the sequence of said target segment;

(2) ligating said annealed first and
35 second segments;

-9-

(first) target segment. If the second target segment is the complement of the first, the 3'-subsegment of the second preferably, but not necessarily, has the same number of nucleotides as the 5'-subsegment of the first. Preferably, the two segments to be ligated, that are employed in the second set of cycles, would be present at a concentration much higher than that of the two segments employed in the first set of cycles; at a minimum, the ratio of the concentration of the segments employed in the second set of cycles to the concentration of the segments employed in the first set of cycles would preferably be the ratio of the concentration of ligated segments resulting from the first set of cycles to the initial concentration of the first target segment. (Note that reference to "concentration," in the singular, for the segments of each pair, is deliberate, as it is assumed in the present specification, unless stated specifically to be otherwise, that the concentrations of the two segments of a pair for annealing to and ligation on a target segment or complement thereof, are equal. While this equality of concentrations is preferred, it is not required to carry out the various aspects of the invention.)

Reference herein to a "nucleic acid" segment means a DNA segment or an RNA segment. Reference to an RNA segment as having the "same sequence" as a DNA segment means that the RNA segment has the same number of ribonucleotides as the DNA segment has 2'-deoxyribonucleotides and that the ribonucleotide at each position in the sequence of the RNA segment has the same base as the 2'-deoxyribonucleotide at the corresponding position in the sequence of the DNA segment, with the exception that, for a ribonucleotide with a uridine at a position in the RNA segment, there is a 2'-deoxyribonucleotide with a thymidine at the corresponding position in the DNA segment.

-11-

(3) strand-separating said target segment from said ligated first and second segments;

(4) annealing to ligated first and second segments (A) a third DNA segment, which has (i) a
5 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the same sequence as said 5'-subsegment of said target segment and (B) a fourth DNA segment, which has (i) a 5'-terminal phosphate and (ii) a 5'-subsegment which terminates with
10 said 5'-phosphate and has the same sequence as said 3'-subsegment of said target segment; and

(5) ligating said annealed third and fourth segments.

This process of the invention, which entails
15 repeating, as often as desired or, for the purpose for which the amplification is carried out, necessary, cycles of (i) annealing, of one pair of DNA segments (e.g., S_1' , S_2') to adjoining subsegments of target segment and a different pair of DNA segments (e.g., S_1'' , S_2'')
20 to adjoining subsegments of complement of target segment, provided that the 5'-subsegment, of the adjoining subsegments of target segment, is the complement of the 3'-subsegment, of the adjoining subsegments of the complement of target segment; (ii) ligating of each
25 annealed pair; and (iii) strand-separation of the ligated pairs from the target segment or complement thereof to which they were annealed, results in an increase in copy numbers of the ligated pairs of segments (and therefore of target segment and complement of target segment) which
30 is approximately exponential as a function of the number of such cycles, involving, in principle, a doubling with each cycle (until the ratio of the concentration of target segment or complement thereof to the concentration of ligase (or co-factor for the ligase, if required) or
35 the ratio of the concentration of target segment or

-14-

minimum, the ratio of the concentration of the segments employed in the second set of cycles to the concentration of the segments employed in the first set of cycles would preferably be the ratio of the concentration of ligated segments resulting from the first set of cycles to the initial concentration of the first target segment.

The invention further includes a process for testing a sample for the presence of a target nucleic acid analyte, which comprises a target segment of known sequence, which process comprises:

(A) amplifying said target segment or the complement of said target segment to a concentration which is detectable, said amplification comprising:

(1) annealing to said target segment
(A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment of said target segment and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment of said target segment, provided that the sequence of a segment which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment is complementary to the sequence of said target segment;

(2) ligating said annealed first and second segments;

(3) strand-separating said target segment from said ligated first and second segments;

(4) annealing to said ligated first and second segments (A) a third DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the same sequence as said 5'-subsegment of said target segment and

-15-

(B) a fourth DNA segment, which has (i) a 5'-terminal phosphate and (ii) a 5'-subsegment which terminates with said 5'-phosphate and has the same sequence as said 3'-subsegment of said target segment;

5 (5) ligating said annealed third and fourth segments; and

(6) to the extent necessary to obtain a detectable concentration of said target segment or its complement,

10 (i) strand-separating ligated third and fourth DNA segments from ligated first and second DNA segments;

(ii) annealing unligated third and fourth DNA segments to ligated first and second DNA segments and unligated first and second DNA segments to ligated third and fourth DNA segments;

15 (iii) ligating said unligated third and fourth DNA segments annealed in accordance with step (6)(ii) and said unligated first and second DNA segments annealed in accordance with step (6)(ii); and

20 (iv) repeating steps (6)(i) to (6)(iii); and

(B) after said amplification process, testing said sample for the presence of said ligated first and second DNA segments or said ligated third and fourth DNA segments.

The amplification and detection processes of the invention can be carried out to amplify or detect simultaneously more than one target segment or complement thereof of known sequence. This can be accomplished by employing simultaneously, in accordance with the processes, for each target segment, a pair of segments which anneal to adjoining subsegments of the target segment and are ligated thereon and, if required for the process of the invention, a pair of segments which anneal

-12-

complement thereof to the concentration of a pair of DNA segments to anneal thereto for ligation becomes so high that the exponential increase cannot be sustained, given the reaction conditions of and time allotted for the
5 annealing and ligation of each cycle).

In this process of the invention, involving annealing and ligation of two pairs of DNA segments simultaneously, it is preferred, although not required, and assumed in the present specification unless stated
10 specifically to be otherwise, that the initial concentrations, at the beginning of the amplification process, of all four unligated segments (two segments in each of two pairs) are the same.

In still another of its aspects, the invention
15 includes a process for testing a sample for the presence of a target nucleic acid analyte, which comprises a target segment of known sequence, which process comprises:

(A) amplifying a DNA segment with the
20 sequence complementary to that of said target segment to a concentration which is detectable by an assay for said DNA segment, said amplification comprising:

(1) annealing to said target segment
(a) a first DNA segment, which has (i) a 5'-terminal
25 phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment of said target segment, and (b) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which
30 terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment of said target segment, provided that the sequence of the segment, which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment
35 is complementary to the sequence of said target segment;

-13-

(2) ligating said annealed first and second DNA segments;

(3) strand-separating said ligated first and second DNA segments from said target segment;

5 and

(4) repeating steps (1), (2), and (3) at least once until a concentration of said ligated first and second DNA segments is obtained which is detectable by an assay for said ligated segments; and

10 (B) after said amplification process, testing said sample for the presence of said ligated first and second DNA segments.

In the process, described in the immediately preceding paragraph, for detecting the presence in a sample of a nucleic acid analyte comprising a segment of known sequence, the amplification process can be carried out first, for a number of cycles, with two segments (e.g., S_1' and S_2') that hybridize to the target segment and then, for an additional number of cycles, with two different segments, which hybridize to adjoining subsegments of the segment made upon ligation of the two segments employed in the initial number of cycles. For the second set of cycles, some subsegment of the segment, $S_2'S_1'$, made upon ligation of the two segments employed in the initial number of cycles is, in effect, a second target segment. This second target segment is preferably, but not necessarily, the complement of the (first) target segment. If the second target segment is the complement of the first, the 3'-subsegment of the second preferably, but not necessarily, has the same number of nucleotides as the 5'-subsegment of the first. Preferably, the two segments to be ligated, that are employed in the second set of cycles, would be present at a concentration much higher than that of the two segments employed in the first set of cycles; at a

15
20
25
30
35

-17-

with the segment's ability to anneal to, and be ligated on, target segment or complement thereof; then, through the label on this segment, amplified target or complement thereof (whichever is formed by ligation of the labeled
5 segment in the amplification process) can be detected and the need for a third container comprising a labeled probe, for amplified target or complement, to provide detectability can be obviated. Containers suitable for kits according to the invention are well known in the art
10 and include, for example, capped or sealed tubes, of glass or plastic of various types, said containers being easy and convenient to open, when it is desired to employ the contents thereof in a process according to the invention, and convenient for the obtaining or dispensing
15 therefrom desired quantities of the reagents they hold.

The amplification processes of the invention depend on the ligation of single-stranded DNAs catalyzed by DNA ligases. Such ligation requires that the DNA strands to be ligated be annealed (i.e., hybridized
20 through complementary base-pairing) to adjoining subsegments of a single-stranded nucleic acid template, such that a 3'-hydroxyl group at the 3'-terminus of one of the strands to be ligated be immediately adjacent a 5'-phosphate at the 5'-terminus of the other of the
25 strands to be ligated. Thus, the amplification processes yield increased copy numbers of target segments, or complements thereof, that are DNAs and that are themselves suitable as templates for DNA ligase-catalyzed ligation of DNA strands annealed thereto.

30 The target segments, on which the amplification processes of the invention are applied, are of known sequence. Further, the target segments and the nucleic acid analytes, which comprise target segments and are detected in the detection processes of the invention, are
35 preferably DNAs.

-18-

The target segments or complements of target segments can be segments of longer DNA strands or can be self-contained. A target segment, or complement thereof, can be present in a sample comprising nucleic acids on which an amplification process according to the invention is to be carried out or can be made in the course of such an amplification process. In particular, a DNA made by ligating, in accordance with the invention, two segments annealed to the complement of a target segment, provides a target segment; and a DNA made by ligating, in accordance with the invention, two segments annealed to a target segment, provides a complement of target segment.

A DNA which is, or which comprises, a target segment which (or the complement of which) can be amplified in accordance with the invention, can be provided by a process which comprises reverse transcription of an RNA which is, or which comprises, a segment with the sequence of the target segment or the complement thereof. Providing DNA for amplification or detection in accordance with the present invention from RNA is particularly advantageously applied to samples wherein substantially all, or at least a substantial fraction, of the nucleic acid with a segment with the sequence of target segment or the complement thereof is RNA. Such samples include, for example, samples of mRNA isolated from cells and separated from the DNA thereof, wherein an RNA which comprises a segment with a particular sequence is of interest, and samples of nucleic acid isolated from cells wherein the segment of interest is a segment of the genome of an RNA virus, such as a retrovirus, which genome is present in an infected cell in DNA (i. e., proviral) form and RNA form. Examples of such viruses are HIV-1 and HIV-2, causative agents of acquired immune deficiency syndrome in humans.

-19-

The skilled are well aware of methods for reverse transcribing RNA, and particularly RNAs of known sequence, employing reverse transcriptases such as those of avian myoblastosis virus and Moloney murine leukemia virus. See, e.g., Ausubel et al., eds., Current
5 Protocols in Molecular Biology, Wiley-Interscience, New York, 1987, pp. 3.7.1 - 3.7.3.

Further, as the skilled are aware, a first DNA, made by reverse transcription of an RNA, can be employed
10 as a template for preparation of a second DNA employing a DNA-dependent DNA polymerase, including E. coli DNA polymerase I, the Klenow Fragment thereof and the avian myoblastosis virus reverse transcriptase (which also has DNA-dependent DNA polymerase activity). See, e.g.,
15 Ausubel et al., supra, at pp. 3.5.1 - 3.5.14 and 3.7.1 - 3.7.3. The second DNA, which has the sequence complementary to that of at least a 3'-segment of the first DNA, and the sequence the same as that of the segment of the RNA reverse transcribed to make said 3'-segment of the
20 first DNA, can also provide a DNA target segment to be amplified or detected in accordance with the present invention.

As shown in the Figures, a target segment S_1S_2 for amplification by the processes of the
25 invention is defined by the sequences of the 5'-subsegment, S_{1C} , of one of the segments (S_1') and the 3'-subsegment, S_{2C} , of the other of the segments (S_2') which, in accordance with the invention, are ligated after annealing to the target segment, said
30 5'-subsegment of segment S_1' having the sequence complementary to that of the 5'-subsegment, S_1 , of the target segment and said 3'-subsegment of segment S_2' having the sequence complementary to that of the 3'-subsegment, S_2 , of the target segment, provided that
35 the target segment consists of said 3'-subsegment and said 5'-subsegment thereof.

-20-

Definition of a target segment also provides the definition of the complement thereof, and vice-versa. Thus, with reference to the Figures, the complement $S_{2C}S_{1C}$ of a target segment for amplification by the processes of the invention is defined by the sequences of the 5'-subsegment, S_2 , of one of the segments (S_2'') and the 3'-subsegment, S_1 , of the other of the segments (S_1'') which, in accordance with the invention, are ligated after annealing to the complement of the target segment, said 3'-subsegment of segment S_1'' being the complement of the 3'-subsegment S_{1C} of complement of target segment and said 5'-subsegment of segment S_2'' being the complement of the 5'-subsegment S_{2C} of complement of target segment, provided that the complement of target segment consists of said 5'-subsegment (S_{2C}) and said 3'-subsegment (S_{1C}) thereof.

As indicated above, ligation of two single-stranded DNA segments by a DNA ligase requires that a 5'-terminal subsegment of one of the segments (with a 5'-terminal phosphate) and a 3'-terminal subsegment of the other of the segments (with a 3'-terminal hydroxyl) be stably hybridized to a single-stranded nucleic acid, preferably DNA, template. All DNA ligases can catalyze ligation of DNA segments hybridized to a DNA template, and some DNA ligases (e.g., the T4 ligase) can also catalyze ligation of DNA segments hybridized to an RNA template, although usually somewhat less efficiently than when the segments are hybridized to a DNA template.

This requirement for stable hybridization of the segments to be ligated is the basis for the ligase-based replication occurring preferentially on a target segment of interest (or the complement thereof) in a complex mixture of nucleic acids, whereby ligase-based replication can be employed to amplify a particular target segment (or complement thereof) in such a mixture. This

-21-

is because, under conditions of sufficient stringency, two DNA segments will hybridize to each other with stability sufficient for DNA-ligase catalyzed ligation at a practically useful rate only if the segments have exactly complementary sequences over subsegments of a sufficient number of bases. (Reference herein to "complementary sequences," without other qualification, means the sequences are exactly complementary, base for base.)

10 Annealing of segments to be ligated to a target segment or complement thereof and the subsequent ligation in accordance with the invention are carried out under conditions of stringency at which the DNA ligase remains active and at which the probability that, if two DNA
15 segments to be ligated on a target segment or two DNA segments to be ligated on a complement of a target segment hybridize to a segment, in immediately adjacent positions in an orientation required, and with stability sufficient, for ligation to be catalyzed by the ligase,
20 that segment, to which the two segments to be ligated have hybridized, is other than a target segment or complement thereof, respectively, is significantly less than 0.5 and preferably less than about 0.1. This probability is referred to as the probability of spurious
25 ligation.

 There are two sources for spurious ligation. Before considering these sources, it must be noted that spurious ligation is a potential problem only when amplification in accordance with the invention is part of
30 a process for detecting whether a particular nucleic acid analyte, of which the target segment or complement thereof or both are at least a part, is present in a sample. In other applications of amplification according to the invention, the probability of spurious ligation
35 is irrelevant.

-22-

The first source of spurious ligation is that segments of DNA may occur in a sample, on which amplification according to the invention is carried out, which have the same sequence as target segment or
5 complement thereof or both but which are not part of the nucleic acid analyte, which is to be detected in the process comprising the amplification.

The second source of spurious ligation is that segments to be ligated might anneal suitably for ligation
10 to segments which differ in sequence from target segment or complement thereof.

With a given DNA ligase and a sample of nucleic acids of given complexity, it is well within the skill of the ordinarily skilled in the art to adjust without
15 difficulty stringency conditions, for hybridizations and during ligations, and lengths of target segments, complements thereof, and the two subsegments of each, to which segments must hybridize to be ligated, in order to maintain the activity of the ligase and hold the
20 probability of spurious ligation ascribable to the second of the sources thereof to an acceptably low level.

Typically, each of the two subsegments of a target segment (e.g., S_1 and S_2 in the Figures), and of the complement thereof (e.g., S_{2c} and S_{1c} in the
25 Figures), must be at least 5 nucleotides in length so that the subsegments, of, e.g., S_1' and S_2' for the target segment and S_1'' and S_2'' for the complement of target segment, which hybridize for ligation to the target and complement thereof, will be hybridized thereto
30 with sufficient stability for the ligation, catalyzed by the DNA ligase, to proceed at an appreciable rate. More preferably, each of these two subsegments of a target segment and these two subsegments of the complement thereof will be at least 8 nucleotides in length. As a
35 practical matter, in view of the value of preparing by an

-23-

in vitro, solid-phase method the segments to be ligated in the amplification processes of the invention, and the current difficulty of preparing large amounts of such segments that are longer than about 100 nucleotides by such methods, each of the two subsegments of a target segment and the two subsegments of the complement of the target, to which segments hybridize for ligation, will have fewer than about 100 nucleotides. In principle, however, these subsegments of target and complement thereof can be much longer, up to at least several thousand nucleotides in length, provided that, if longer than about 150 nucleotides, the corresponding segments to be hybridized for ligation will preferably be prepared by processes comprising DNA cloning in vivo, as in *E. coli*.

While the minimum length of a target segment (and complement thereof) is 10 nucleotides, and preferably at least 16 nucleotides, as discussed above, primarily to ensure sufficiently stable hybridizations for DNA-ligase catalyzed ligation, longer targets (and complements) are indicated in certain circumstances, when the amplification according to the invention is part of a process for detecting the presence of a particular nucleic acid (e.g., gene or segment thereof, cDNA, mRNA) in a sample, in order to reduce the probability of spurious ligation discussed above. Thus, with regard to the first of the aforementioned sources for this spurious ligation, the shorter a target segment is, the more likely it is that a segment, which has the same sequence as the target segment but is not part of the nucleic acid analyte sought to be detected, occurs in a sample. The probability that a segment with the same sequence as a target segment (or complement thereof) occurs in a nucleic acid other than the one sought to be detected in a sample increases with the complexity of the nucleic acid of a sample. Thus, for example, nucleic acids from

-24-

cells of an eukaryotic organism, such as a human or other mammal, with a complex genome are more likely to have such spurious segments than nucleic acids from organisms, such as bacteria or yeasts, with simpler genomes.

5 Generally, for a target segment at least about 30 nucleotides in length, it is unlikely that a segment of the same sequence in a nucleic acid other than the one sought to be detected will be present in a sample prior to amplification according to the invention, even in the
10 most complex of nucleic acid samples.

Of course, for reasons of convenience or because a sequence of 30 or more nucleotides in a continuous stretch is not known for a nucleic acid sought to be detected, it may be desirable or necessary to employ a
15 target segment (and, of course, the complement thereof) with fewer than 30 nucleotides. For a target segment of any length, "suitability" means the absence, prior to amplification according to the invention, in a sample to be analyzed for the presence of a nucleic acid analyte,
20 of a significant incidence of segments of the same sequence as target segment (or complement thereof, if amplification with production of both target segment and complement is carried out) in nucleic acids other than nucleic acid analyte (i.e., the specific nucleic acid,
25 the presence of which is being assayed for in the sample). The incidence of such segments would be significant if the copy number of such segments, in nucleic acids other than nucleic acid analyte sought to be detected, is so high that background signal in an
30 assay for the nucleic acids to be detected would unavoidably be unacceptably high. Suitability of a target segment can be ascertained readily by the skilled. For example, when the target segment has the sequence of part of a gene of a virus, such as an HIV-1,
35 HIV-2, hepatitis B virus or any of many others, which

-26-

in certain pathological conditions at much higher concentrations.

Any DNA ligase can be employed to catalyze the ligation steps in the processes of the invention.

5 Preferred, however, are ligases, such as that of *E. coli*, which normally have little or no activity in catalyzing ligation of blunt-ended, double-stranded fragments. Any ligase that is used is preferably used under conditions at which catalysis of such blunt-ended ligation, if it
10 occurs at all, occurs at a rate at least 10^3 , and more preferably at least 10^6 , times more slowly than ligation of annealed single strands. Ascertaining such conditions for any particular ligase is easily within the skill of a person of ordinary skill in the art.

15 DNA ligases that are stable in solution above about 90 °C, that have significant catalytic activity above at least about 50 °C in ligating single-stranded DNA segments hybridized to adjoining subsegments of a DNA template, and that have insignificant (i.e., at least
20 10^3 times slower than said single-stranded ligation rate) blunt-ended ligation activity above about 50 °C would be preferred for carrying out the methods of the present invention. With such enzymes, strand-separations by heating in the course of amplification in accordance
25 with the invention could be carried out without need for replenishing enzyme prior to each ligation step and ligations could be carried out at higher temperatures, and, consequently, higher stringencies, thereby increasing the specificity of amplification for target
30 segment (or complement thereof) by reducing spurious ligations due to the second source, as described above. Such thermally stable DNA ligases exist in thermophilic organisms, such as thermophilic bacteria that exist in and around hot springs; and the isolation of such ligases
35 from such microorganisms would be straightforward for the person of ordinary skill.

-27-

The preferred application of processes according to the invention for amplifying a DNA segment is to facilitate the detection of such segment, and therefore, indirectly, a nucleic acid or segment thereof in which a segment of the same or complementary sequence occurs, by nucleic acid probe hybridization assay methods.

The amplification processes of the invention do, however, have other applications. For example, the processes can be employed to make large quantities of a single-stranded DNA with a sequence complementary to that of all, or a segment, of a DNA (or RNA) made in a small quantity by an in vitro, solid phase method. Further, the processes can be employed to make large quantities of a double-stranded DNA, the sequence of one strand of which is the same as that of all, or a segment, of a DNA (or RNA) made by an in vitro, solid phase method or of all, or a segment of, an mRNA of interest. Double-stranded DNAs prepared by amplification processes of the invention can, by methods well understood in the art, be ligated into appropriate vectors for transcription to yield large quantities of RNA of interest or for cloning to yield even larger quantities of the double-stranded DNA or ligated into a gene, on vectors, to effect mutations of interest in the gene.

As indicated in the Figures, and in the Example, a DNA segment for ligation in the course of amplification in accordance with the invention may have a "tail," a terminal subsegment which, for lack of sufficient sequence complementarity, does not anneal to target segment (or complement thereof). It is preferred that at most one of the two or four DNA segments that are ligated in the course of amplification in accordance with the invention have such a tail. If two or more of the segments have such tails, it is preferred that the sequences of the tails be such that no pair of tails

-28-

anneal stably under the conditions employed to cause the segments to anneal, prior to ligation, to target segment or complement thereof; most preferably the sequence of each of the tails will be non-complementary to that of the other tails and the target segment and its complement (i.e., no sequence of more than three nucleotides in a tail will be complementary to any sequence of more than three nucleotides in any of the other tails or in the target segment or its complement).

5 A tail can serve a number of functions. For example, if the double-stranded DNA resulting from an amplification process according to the invention is to be ligated in a particular direction into a vector between two cleavage sites, one for each of two restriction enzymes with differing cleavage sites, at least one of which leaves an overhanging end, a tail can be included on one (or two) of the segments ligated in the course of the amplification process so that the double-stranded DNA resulting from the amplification will have overhanging end(s) with sequence(s) complementary to those of the overhanging end(s) of the cleaved vector and ligation of the double-stranded DNA into the vector can be carried out by standard techniques. For example, with reference to the Figures, if a double-stranded segment from amplification is to be ligated into well known plasmid pBR322 between the EcoRI and BamHI sites thereof, a tail, of sequence 5'-pAATT-3', would be included as the 5'-subsegment of one of S_2' and S_1'' , and a tail, of sequence 5'-pGATC-3', would be included as the 5'-subsegment of the other of S_2' and S_1'' . For ligation between the EcoRI and PvuII sites of pBR322, only the tail of sequence 5'-pAATT-3' on one of S_2' and S_1'' would be used.

35 A tail, such as of poly(dC) or poly(dG), or poly(dA) or poly(dT) if the sample of nucleic acid on

-29-

which the amplification process is carried out does not have an appreciable amount of polyadenylated RNA, on one of the segments ligated in the course of amplification in accordance with the invention, can facilitate isolation of the DNA product of the amplification, by well known techniques employing affinity chromatography over a column derivatized with polymers of the ribonucleotide or 2'-deoxyribonucleotide that is the complement of the 2'-deoxyribonucleotide in the tail. As illustrated in the Example, such a tail can also facilitate capture of one strand of the product of the amplification on a solid support, such as nitrocellulose paper, preparatory to detection of the strand with a nucleic acid probe.

One or more of the segments ligated in the course of amplification in accordance with the invention can be labeled for detection in any way known in the art which does not interfere with their ability to be ligated via catalysis by a DNA ligase or with their ability, once ligated, to serve as template for ligation of unligated segments of complementary sequence. Such labeling facilitates detection of the DNA resulting from amplification, provided that, after the amplification process, excess, unligated and labeled segments are separated from ligated, labeled segments. Such separation can be accomplished by any of a number of methods known in the art, including various electrophoretic and chromatographic methods. See, e.g., Ausubel et al., supra, at pp. 2.5.1 - 2.8.1; Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, 1986, Chapter 10.

Preferred methods of labeling the segments are with a radioactive isotope of one of the elements that occurs in nucleotides, most preferably ^{32}P , or, provided the segment is not one which requires a 5'-terminal phosphate group to effect proper ligation

-30-

with another of the segments to be ligated during amplification, with a biotin linked through an alkylamine linker arm and a phosphoramidate group to the 5'-carbon of the segment. A segment, particularly one which, as preferred, is an oligonucleotide made by an in vitro, automated solid-phase method, is labeled with ^{32}P preferably by kinasing by a standard technique with T4 polynucleotide kinase and gamma- $^{32}\text{PO}_4$ -labeled ATP. A biotin-derivatized segment is preferably prepared by the method of Chu and Orgel, DNA 4, 327-331 (1985). Methods of detecting DNAs labeled with ^{32}P , by means of radiation due to radioactive decay of the isotope, or biotin are well known to those of ordinary skill in the art. Biotin-labeled DNA is preferably detected by means of a chromogenic chemical reaction catalyzed by an enzyme, such as an alkaline or acid phosphatase, a peroxidase or a beta-galactosidase, that is conjugated to avidin or streptavidin and then joined to the biotin via a complex formed by the high affinity binding of biotin to the avidin or streptavidin.

The DNA prepared by amplification in accordance with the invention, if not labeled, can nonetheless be detected in any nucleic acid probe hybridization assay method known in the art, including such methods employing DNA from the amplification immobilized on a solid support, such as a nitrocellulose or nylon filter. See, e.g., Meinkoth and Wahl, Anal. Biochem. 138, 267-284 (1984). Any single-stranded nucleic acid that is labeled for detection and capable, in the hybridization assay system, to hybridize with sufficient specificity to one strand or the other of the DNA made in the amplification (if double-stranded DNA is made), or the strand that is made (if only one is made), can be employed as the probe to detect the DNA made in the amplification. A preferred probe, however, will have the sequence of the amplified

-31-

target segment, or the complement thereof, and will be labeled either with $^{32}\text{PO}_4$ at the 5'-terminus or with biotin linked through an alkylamine linker arm and a phosphoramidate group to the 5'-carbon of the segment as described by Chu and Orgel, DNA 4, 327-331 (1985).

Samples, on which the processes of the invention are carried out, are aqueous solutions of nucleic acids. Such samples are prepared by standard techniques from viruses or cells from a culture, a sample of body fluid such as blood, lymph, ascites fluid, milk or the like, a sample of a foodstuff, feces, a tissue sample, or the like. An aqueous solution of nucleic acid, suitable for application of the processes of the invention, can also be readily prepared by standard methods with nucleic acid prepared by an in vitro method, such as an automated, solid-phase method.

If DNA comprising a target segment is to be made by a process comprising reverse transcription of RNA, the RNA will be retained in the solution until, at least, the reverse transcription has been carried out. RNA can be retained in a solution on which the amplification and other processes of the invention are carried out. Alternatively, the RNA can be removed, by degradation by treatment with ribonucleases or base or by other known methods, so that the polynucleotides that remain in such a solution are substantially only DNAs.

Typically, an amplification process of the invention is initiated on a solution of DNAs, at about 1 $\mu\text{g/ml}$ to about 100 $\mu\text{g/ml}$ concentration, in a ligation buffer. The ligation buffer is an aqueous solution at a pH between about 7 and about 9, at which the DNA ligase to be used is active, maintained by any standard buffer which the ligase can tolerate (preferably Tris-HCl at a concentration of 5 mM to 50 mM); a small amount of EDTA, typically at 0.1 to 10 μM ; Mn^{+2} or, preferably, Mg^{+2}

-32-

required for DNA ligase activity, preferably as the chloride salt at 0.2 mM to 20 mM concentration; any co-factor required for ligase activity (DPN (otherwise referred to in the art as "NAD⁺") in the case of the E. coli ligase and ATP in the case of the T4 ligase) at a concentration between about 1 μ M and 100 μ M; a reducing agent such as dithiothreitol or dithioerythritol at about 0.1 mM to about 10 mM if (as in the case of the T4 ligase) necessary for suitable activity of the ligase being employed; and the segments to be ligated in the amplification process of the invention (with reference to the Figures, either S₁' and S₂' or, in addition to those two, also S₁" and S₂") at a very large molar excess, typically 10⁸ to 10¹², relative to the anticipated concentration of target segment present in the solution before initiation of the amplification process. A concentration between about 1 nM and 1 μ M for each of these segments will usually assure that a sufficient molar excess, relative to the concentration of target segment, is present.

Alternatively, the final step in preparation of ligation buffer can correspond to the initiation of amplification process in accordance with the invention. In this alternative, the DNA segments to be ligated are dissolved in a first buffer, which has the same composition as ligation buffer except that it lacks the DNA sample to be subjected to amplification, and the DNA sample to be subjected to amplification is dissolved in a second buffer, which has the same composition as ligation buffer except that it lacks the segments to be ligated. Then ligation buffer is made by combining the first buffer solution, immediately after treatment, if necessary, by heating or another process to render the segments to be ligated single-stranded, with the second buffer, immediately after it also has been treated, if

-33-

necessary, to render single-stranded any DNA with target segment.

If the ligation buffer is made by dissolving directly in the same solution both sample DNA and the segments to be ligated, without combining separate solutions of the sample DNA and segments to be ligated in a manner similar to that just described, and if either the target segment sought to be subjected to amplification according to the invention, if it is present in the sample DNA, occurs as part of double-stranded DNA or two pairs of segments, rather than one pair, are to be ligated in the amplification process, then, as will be apparent to the skilled in the art, immediately prior to the first annealing step of the amplification process according to the invention, the DNA in the ligation buffer must be treated to strand-separate double-stranded DNA therein into single-stranded form.

Although enzymes, e.g., helicases, are known which can catalyze such strand-separation under certain conditions, strand-separations, for purposes of the present invention, are effected by simply heating the DNA solution to a temperature above the melting temperature for strand-separation of the double-stranded DNA of the solution. Heating a solution to above 80 °C - 90 °C and holding at the temperature for more than a few seconds (preferably more than about a minute) is adequate to effect strand-separation of DNAs in a ligation buffer.

Once the necessary DNAs are in single-stranded form in the ligation buffer, the first annealing step of the amplification process is carried out. This is accomplished by simply cooling the solution buffer to a temperature near or somewhat below the melting temperature for the duplexes to be formed between the segments to be ligated and the subsegments of target segment (or complement thereof) to which those segments

-34-

must hybridize stably for ligation to be catalyzed. It is preferred that this temperature also be in the range of temperatures at which the ligase to be employed retains significant activity. In a preferred procedure, 5 the annealing occurs by plunging the tube holding the solution into a bath (e.g., an ice bath) at about 0 °C, holding the tube in such bath for a few seconds, and then placing the tube in another bath maintained at the temperature at which the ligation reaction is to be 10 carried out. If maintaining stringency at a high enough level to reduce spurious ligation due to the second cause, as discussed above, requires annealing at a temperature above that at which the ligase retains significant activity, the tube, preferably after being 15 held at 0 °C for a few seconds, is placed in a bath at this higher temperature until the solution reaches that temperature and then placed into a third bath at a temperature which is suitable for catalysis by the ligase.

20 Once the solution reaches a suitable temperature for catalysis of ligation by the DNA ligase, an aliquot of ligase solution, of preferably significantly smaller volume than (i.e., between about 0.1 and 0.001 times) that of the ligation buffer in which the initial 25 annealing is carried out, is added to the ligation buffer and, thereby, the ligation initiated. The ideal duration of a ligation reaction can be estimated readily by the skilled in the art, and will depend on several factors, including the particular ligase employed, the 30 concentration of the ligase, the activity of the ligase at the temperature, pH, ionic strength, and Mg^{++} or Mn^{++} concentration in the solution in which the ligation is occurring, and concentrations of target segments (and complements thereof) and segments to be 35 ligated in the ligation reaction. Typically, it will be

-35-

adequate and desirable to allow the ligation reaction to continue for between about 1 minute and 30 minutes, when *E. coli* DNA ligase is used, at about 0.001 to about 0.1 "Modrich-Lehmann" unit of enzyme per μ l of solution at
5 between about 20 °C and 40 °C, in a typical ligation buffer as described above.

With a thermostable ligase that retains single-stranded DNA ligation activity at higher temperatures, as discussed above, higher temperatures,
10 above about 50 °C or even 60 °C, would be employed in the ligation step, provided that the temperature permits hybridization, of segments to be ligated to their respective templates in the ligation solution, that is sufficiently stable for ligation to occur at a suitable
15 rate.

After the period for the ligation reaction, the reaction is terminated by inactivating the ligase, preferably by raising the temperature of the solution to a temperature at which the ligase is essentially
20 inactive. In the case of the *E. coli* DNA ligase, complete inactivation can be achieved by a few seconds at above 75 °C.

After the ligation reaction, the DNA of the solution is strand-separated. In a preferred procedure,
25 at the conclusion of the time allowed for the ligation reaction, the temperature of the solution is raised high enough so that the DNA of the solution is strand-separated. This raising of the temperature is conveniently accomplished by simply dipping the tube with
30 the ligation buffer into a bath maintained at a high enough temperature. When the ligation buffer has a composition in the ranges indicated above, even if the composition is modified slightly by the addition, along with DNA ligase, of solution that might differ in
35 composition, holding at between about 80 °C and 100 °C

-36-

for more than a few seconds (and preferably more than about a minute) suffices for strand-separation of the DNA.

Then, as often as necessary (e.g., to amplify a target segment or complement thereof to a concentration that is detectable (i.e., measurable above background, established by suitable controls) by a nucleic acid probe hybridization assay method that is to be employed after the amplification) or desirable, reannealing can be carried out as described above for the annealing step, ligation can be carried out after the reannealing by adding another aliquot of DNA ligase solution (if the ligase was denatured and irreversibly inactivated in the preceding strand-separation step) and incubating the solution as described above for the ligation step, a strand-separation can be carried out as described above after the ligation, and the annealing-ligation-strand-separation cycle can be started again.

After the amplification, the DNA in the final ligation buffer can be assayed, for ligated segments S_1' and S_2' , comprising together a subsegment with the sequence complementary to that of target segment, or, if two pairs of segments to be ligated were employed, ligated segments S_1'' and S_2'' , comprising together a subsegment with the sequence of target segment, by any of a large number of nucleic acid probe hybridization assay methods known in the art. In whichever of these methods is employed, a probe which comprises a subsegment with either the sequence of target segment (if $S_2'S_1'$ is to be detected) or the sequence of the complement of target segment (if $S_1''S_2''$ is to be detected) is preferably employed. Alternatively, at least one of the segments to be ligated in the course of amplification in accordance with the invention can be labeled for detection, in a manner which does not interfere with its

-37-

ability to hybridize to a template appropriately to be ligated, and then labeled $S_2'S_1'$ or $S_1''S_2''$ resulting from amplification can be detected.

5 All of the manipulations required to carry out the methods of the invention, including combining solutions, changing temperatures, blotting DNA and the like, can optionally be carried out automatically with appropriate devices rather than manually.

10 The invention will now be illustrated with the following Example.

EXAMPLE

AMPLIFICATION AND DETECTION OF A SEGMENT OF THE GENE FOR THE BETA-SUBUNIT OF SICKLE-CELL HEMOGLOBIN

15

DNA is extracted from human white blood cells as follows, following Geever et al., Proc. Natl. Acad. Sci. (USA) 78, 5081-5085 (1981) and Goosens and Kan, Meth. Enzymol., 76, 806 (1981):

20

A 10 ml blood sample is collected into a vacutainer containing anticoagulant and stored at -20°C until used. The blood cells are pelleted by centrifugation at $2000 \times g$. The plasma is discarded and the cells washed with 0.9% NaCl. Reticulocytes and older erythrocytes are hemolyzed by the addition of two cell pellet volumes of sterile H_2O . The lymphocytes are collected by centrifugation at $2000 \times g$ for 15 mins. The cells are then dispersed into a 100-ml flask and diluted to 50 ml with buffer of 100 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, 0.5% SDS, and 100 $\mu\text{g/ml}$ proteinase K. This mixture is incubated overnight in a rotating water bath at $50-55^{\circ}\text{C}$ and the nucleic acids are then extracted with a mixture of phenol: CHCl_3 :isoamyl alcohol (25:24:1). The nucleic acids in the aqueous phase are then precipitated with ethanol at -20°C , the precipitate washed with

25
30
35

-38-

70% ethanol and resuspended in TE buffer (20 mM Tris-HCl, 10 mM EDTA, pH 7.4). The nucleic acid solution is then treated with 100 µg/ml ribonuclease. After incubation for 2 hours at 37°C, SDS (to a final concentration of 0.5%) and proteinase K (to a final concentration of 100 mg/ml) are added and the resulting solution is incubated for another hour at 50°C. The mixture is then extracted again with phenol and the DNA precipitated with ethanol as described above. The DNA is suspended in 1-2 ml of TE buffer and dialyzed against 1 mM Tris, pH 7.5, 0.1 mM EDTA for 2 days, with one change of the dialysate solution. The DNA concentration in the sample is determined by measuring the optical density of appropriate dilutions at 260, 280 and 330 nm. About 250 micrograms of DNA is obtained.

The segment of the gene for the beta-subunit of human, sickle-cell hemoglobin to be amplified, if present in the sample, has the sequence:

5'-CTGACTCCTGTGGAGAAGTC-3'.

In the gene for the mutant beta-subunit, which gives rise to sickle-cell anemia (Geever et al., supra), the T at position 11 of the foregoing segment replaces an A in the normal gene, causing the occurrence of a valine in the mutant subunit in place of a glutamic acid as the sixth amino acid from the amino terminus.

The following 10-mers, 9-mer, 15-mer, and 20-mer are synthesized by the phosphoramidite method using an Applied Biosystems DNA Synthesizer:

30	S ₁₁ ' :	5'-CAGGAGTCAG
	S ₁₂ ' :	5'-GACTTCTCCA
	S ₁₁ " :	5'-CTGACTCCTG
	S ₁₂ " (5'-ss) :	5'-TGGAGAAGT
35	G ₅ S ₁₂ ' :	5'-GGGGGACTTCTCCA
	S ₁₂ 'S ₁₁ ' :	5'-GACTTCTCCACAGGAGTCAG

-39-

The above oligomers (approx. 50 O.D.'s) are purified by HPLC on RPC-5, eluting with a perchlorate gradient at pH 12, following Chu and Orgel, Proc. Natl. Acad. Sci. (USA) 82, 963-967 (1985).

5 50 O.D.'s of poly(dC), of average length 300 bases, purchased from Pharmacia, Inc., Piscataway, New Jersey, USA, is combined with 50 mM ATP and 100 units of T4 polynucleotide kinase in 100 ml buffer of 50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.1 mM spermidine and 0.1 mM EDTA at 37 °C for 1 hour. The reaction mixture is then extracted with phenol:CHCl₃ (1:1) and the polynucleotide is obtained from the aqueous fraction by precipitation with EtOH.

15 5 O.D.'s of 5'-phosphorylated-polydC (approx. 1.9 nmoles) and equivalent molar amounts of S₁₂"(5'-ss) and G₅S₁₂' are treated overnight at 15 °C with 1000 Weiss units/ml of T₄ DNA ligase in buffer containing 50 mM Tris, pH 7.8, 10 mM MgCl₂, 1 mM ATP and 20 mM DTT.

20 The ligated product, designated S₁₂", is separated from starting materials on a 6% polyacrylamide denaturing gel. The product is extracted from the gel with buffer containing 500 mM ammonium acetate, pH 7.5, 0.1 mM EDTA and 0.01% SDS, and purified by passage through a Du Pont NensorbTM nucleic acid purification cartridge purchased from DuPont Co., Wilmington, Delaware, USA.

30 2 O.D.'s of S₁₁' and S₁₂'S₁₁' and 5 O.D.'s of S₁₂" are treated, separately, for 1 hour at 37 °C with 50 mM ATP and 100 units of T4 polynucleotide kinase in 100 µl of buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA. Each reaction mixture is then extracted with phenol:CHCl₃(1:1) and dialyzed against 2 l of H₂O for 1-1/2 hours. The 5'-phosphorylated oligomers of S₁₁' and S₁₂'S₁₁' are then separated from any unphosphorylated starting material by

35

-40-

HPLC on RPC-5 at pH 12, eluting with a perchlorate gradient.

The 5'-phosphorylated $S_{12}'S_{11}'$ is converted to a 5'-biotin hexylamine adduct as follows, following

5 Chu and Orgel, DNA 4, 327-331 (1985):

3 O.D.'s of 5'-phosphate derivatized

$S_{12}'S_{11}'$ is reacted with 0.15 M 1-ethyl,3,3-dimethyl-aminopropyl carbodiimide (CDI) and 0.4 M hexanediamine in 0.1 M 1-methyl-imidazole buffer, pH 7, for 3 hours at
10 50 °C. The 5'-hexylaminophosphoramidate-derivatized oligomers are purified and separated from unconverted starting material by HPLC on RPC-5 at pH 12 using a perchlorate gradient.

The 5'-hexylaminophosphoramidate-derivatized
15 oligomer is next treated with 5 mg/ml of N-hydroxy-succinimidobiotin in 0.2 M HEPES buffer at pH 7.7 for 1 hour at room temperature. The 5'-biotin-hexylamino-phosphoramidated oligomer is then purified by HPLC on an RPC-5 column at pH 12 using a perchlorate gradient.

20 The amplification procedure is as follows:

The initial ligation reactions are carried out in 100 μ L volumes in Eppendorf tubes at 30 °C. The ligation buffer is of 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 μ M EDTA, and 10 μ M DPN, the co-factor required
25 for E.coli DNA ligase activity.

A sample of the genomic DNA (about 1 μ g, containing about 3×10^5 target segments), isolated from human blood cells as described above, is dissolved in 100 μ L of ligation buffer and enough 5'-phosphorylated
30 S_{11}' , 5'-phosphorylated S_{12}'' , S_{12}' and S_{11}'' are added to bring the solution to 5×10^{-9} M in each. The solution is heated rapidly to 85°C and maintained at that temperature for 2 minutes to cause strand-separation. The solution is then cooled rapidly in iced water and
35 subsequently warmed to 30°C for annealing of segments

-41-

with complementary sequences. Then approximately 1 "Modrich-Lehmann" unit of E.coli DNA ligase (1 μ l of E.coli DNA ligase solution as supplied by the manufacturer, Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA; see Moderich and Lehmann, supra) is added. The reaction mixture is incubated for 10 minutes at 30 °C, and the reaction terminated by heating rapidly to 85 °C.

The reaction is then cycled 19 additional times, as follows:

After 2 minutes at 85 °C, the sample is cooled rapidly to 0 °C, and then warmed to 30 °C. Approximately 1 unit of E.coli DNA ligase is again added (again in approx. 1 μ l of solution) and the reaction mixture incubated for 10 minutes at 30 °C. The reaction mixture is heated rapidly to 85 °C.

After the 20 cycles, an increase in copy number of the 20-base segment of the sickle-cell beta-globin gene of about 10^3 -fold is achieved if the segment is present in the sample, with less than a 10-fold increase in copy number of any other segment in the DNA sample, thus providing an amplification of nearly 10^3 -fold of the segment of interest.

The amplified DNA of the sample is then detected as follows:

The mixture after amplification is diluted to 200 μ L to give a final solution containing 10 mM Tris, 1 mM EDTA, 100 mM NaCl at pH 7.5. Then 20 μ L of 3 M NaOH are added and the solution is incubated for 30 minutes at 60°-70°C. After cooling, the solution is neutralized with 200 μ l of 2 M ammonium acetate, pH 7.0. The DNA is slot-blotted onto nitrocellulose paper that has been prewetted with H₂O and 1 M ammonium acetate, using a minifold slot blotter (Schleicher and Schuell, Inc., Keene, New Hampshire, USA). The papers are then baked in a vacuum oven at 80°C for 1 hour.

-42-

Then the nitrocellulose blots are pre-hybridized for 1 hour at 30°C in hybridization buffer (900 mM NaCl, 6 mM EDTA, 90 mM Tris, pH 7.5, 0.1% SDS) containing 100 ng/ml randomly cleaved RNA and 0.5% Nonidet P-40.

- 5 Hybridization with 1 ng/ml of 5'-biotin-hexylamino-phosphoramidate-derivatized S₁₂'S₁₁', prepared as described above, is then carried out at 55°C for 1 hour.

The blots are then washed with 3 x 25 ml of buffer containing 180 mM NaCl, 10 mM Na₂HPO₄, 1 mM
10 EDTA, 0.1% SDS, pH 7.7, at room temperature. After drying, the filters are developed colorimetrically for 3 hours using an avidin/alkaline phosphatase DNA detection kit purchased from Bethesda Research Laboratories (Gaithersburg, Maryland, USA).

- 15 A detectable signal is obtained, provided that the individual from whom the blood was taken is heterozygous or homozygous for the sickle-cell beta hemoglobin subunit gene sequence indicated above and, consequently, is at least susceptible to sickle-cell
20 anemia.

If no detectable signal is obtained, amplification has not occurred and, provided that the amplification and detection systems are operative (which is ascertainable by running appropriate controls in
25 parallel, as understood in the art), it can be concluded that the individual lacks the sickle-cell sequence and cannot suffer from sickle-cell anemia.

While the invention has been described with some specificity in the present application, the person of
30 ordinary skill in the pertinent art will recognize numerous modifications and variations that are within the spirit of the invention. It is intended that such modifications and variations are within the scope of the invention described and claimed herein.

- 35 Further, various features of the invention are described in the following claims.

-43-

WHAT IS CLAIMED IS:

1. A process for amplifying a DNA segment with the sequence complementary to that of a target DNA segment of known sequence, which process comprises:

5 (1) annealing to said target segment (A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment, of at least 5 nucleotides, of
10 said target segment, and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment, of at least 5 nucleotides, of said target segment, provided
15 that the sequence of the segment, which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment, is complementary to the sequence of said target segment;

(2) ligating said annealed first and
20 second DNA segments;

(3) prior to repeating step (1), strand-separating said target segment from said ligated first and second DNA segments; and

(4) repeating at least steps (1) and (2)
25 at least once.

2. A process according to Claim 1 wherein the strand-separations are carried out by heating and the ligations are catalyzed by E.coli DNA ligase.

3. A process according to Claim 2 wherein both
30 said 5'-subsegment of said first DNA segment and said 3'-subsegment of said second DNA segment are at least 8 nucleotides in length.

4. A process according to Claim 3 wherein said
5'-subsegment of said first DNA segment and said
35 3'-subsegment of said second DNA segment are 100 or fewer nucleotides in length.

-44-

5. A process according to Claim 4 wherein said first DNA segment consists of said 5'-subsegment thereof and said second DNA segment consists of said 3'-subsegment thereof.

5 6. A process according to Claim 4 wherein (1) said first DNA segment consists of (a) the 5'-subsegment thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 3'-terminus of the 5'-subsegment or (2) said second DNA segment consists of (a) the 3'-subsegment
10 thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 5'-terminus of the 3'-subsegment or (3) both (1) and (2) obtain, provided that that, if both (1) and (2) obtain, both of said tails are of dC or both of said tails are of dG.

15 7. A process for testing a sample for the presence of a target DNA analyte, which comprises a target segment of known sequence, which process comprises:

(A) amplifying a DNA segment with the
20 sequence complementary to that of said target segment to a concentration which is detectable by a nucleic acid probe hybridization assay for said DNA segment, said amplification comprising:

(1) annealing to said target segment
25 (a) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment, of at least 5 nucleotides, of said target segment, and (b) a second DNA
30 segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment, of at least 5 nucleotides, of said target segment, provided that the sequence of the segment, which
35 consists of said 5'-subsegment of said first DNA segment

-45-

ligated to said 3'-subsegment of said second DNA segment is complementary to the sequence of said target segment;

(2) ligating said annealed first and second DNA segments;

5 (3) strand-separating said ligated first and second DNA segments from said target segment; and

(4) repeating steps (1), (2), and (3) at least once until a concentration of said ligated first and second DNA segments is obtained which is detectable by said nucleic acid probe hybridization assay; and

(B) after said amplification process, testing said sample for the presence of said ligated first and second DNA segments by said nucleic acid probe hybridization assay.

8. A process according to Claim 7 wherein DNA, which comprises the target segment to be amplified, is made, prior to the amplification process, by a process which comprises reverse transcription of RNA which comprises a segment with the sequence of said target segment or the sequence complementary to that of said target segment.

9. A process according to Claim 7 wherein the strand-separations are carried out by heating and the ligations are catalyzed by E. coli DNA ligase.

10. A process according to Claim 8 wherein the strand-separations are carried out by heating and the ligations are catalyzed by E. coli DNA ligase.

11. A process according to Claim 9 wherein both said 5'-subsegment of said first DNA segment and said 3'-subsegment of said second DNA segment are at least 8 nucleotides in length.

12. A process according to Claim 10 wherein both said 5'-subsegment of said first DNA segment and said 3'-subsegment of said second DNA segment are at least 8 nucleotides in length.

-46-

13. A process according to Claim 11 wherein said 5'-subsegment of said first DNA segment and said 3'-subsegment of said second DNA segment are 100 or fewer nucleotides in length.

5 14. A process according to Claim 12 wherein said 5'-subsegment of said first DNA segment and said 3'-subsegment of said second DNA segment are 100 or fewer nucleotides in length.

10 15. A process according to Claim 13 wherein said first DNA segment consists of said 5'-subsegment thereof and said second DNA segment consists of said 3'-subsegment thereof.

15 16. A process according to Claim 14 wherein said first DNA segment consists of said 5'-subsegment thereof and said second DNA segment consists of said 3'-subsegment thereof.

20 17. A process according to Claim 13 wherein (1) said first DNA segment consists of (a) the 5'-subsegment thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 3'-terminus of the 5'-subsegment or (2) said second DNA segment consists of (a) the 3'-subsegment thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 5'-terminus of the 3'-subsegment or (3) both (1) and (2) obtain, provided that that, if both (1) and (2) obtain, both of said tails are of dC or both of said tails are of dG.

25 18. A process according to Claim 14 wherein (1) said first DNA segment consists of (a) the 5'-subsegment thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 3'-terminus of the 5'-subsegment or (2) said second DNA segment consists of (a) the 3'-subsegment thereof and (b) a poly (dC) tail or a poly (dG) tail ligated to the 5'-terminus of the 3'-subsegment or (3) both (1) and (2) obtain, provided that that, if both (1) and (2) obtain, both of said tails are of dC or both of said tails are of dG.

-47-

19. A process according to Claim 13 or 14 wherein at least one of said first DNA segment and said second DNA segment is labeled with ^{32}P and said detection comprises detection of the decay of said ^{32}P label of said labeled DNA segment or segments.

20. A process according to Claim 13 or 14 wherein said second DNA segment is labeled through its 5'-terminus with biotin and said detection comprises detection of the biotin of said labeled DNA segment through a reaction catalyzed by an enzyme conjugated to avidin or streptavidin complexed with said biotin label.

21. A process according to Claim 20 wherein said enzyme conjugated to avidin or streptavidin is an alkaline phosphatase.

22. A process for amplifying a target DNA segment of known sequence or the complement of said target segment which process comprises:

(1) annealing to said target segment (A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment, of at least 5 nucleotides, of said target segment and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the sequence complementary to that of a 3'-subsegment, of at least 5 nucleotides, of said target segment, provided that the sequence of a segment which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment is complementary to the sequence of said target segment;

(2) ligating said annealed first and second segments;

(3) strand-separating said target segment from said ligated first and second segments;

-48-

(4) annealing to ligated first and second segments (A) a third DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the same sequence as said 5'-subsegment, of at least 5 nucleotides, of said target segment and (B) a fourth DNA segment, which has (i) a 5'-terminal phosphate and (ii) a 5'-subsegment which terminates with said 5'-phosphate and has the same sequence as said 3'-subsegment, of at least 5 nucleotides, of said target segment; and

(5) ligating said annealed third and fourth segments.

23. A process according to Claim 22 wherein the strand-separations are carried out by heating and the ligations are catalyzed by E.coli DNA ligase.

24. A process according to Claim 23 wherein said 5'-subsegment of said target segment and the said 3'-subsegment of said target segment are at least 8 nucleotides in length.

25. A process according to Claim 24 wherein both said 5'-subsegment of said target segment and said 3'-subsegments of said target segment are 100 or fewer nucleotides in length.

26. A process according to Claim 25 wherein said first DNA segment consists of said 5'-subsegment thereof, said second DNA segment consists of said 3'-subsegment thereof, said third DNA segment consists of said 3'-subsegment thereof, and said fourth DNA segment consists of said 5'-subsegment thereof.

27. A process according to Claim 25 wherein at least one of said first, second, third and fourth DNA segments consists of a poly(dC) tail or a poly(dG) tail ligated to: (i) the 3'-terminus of the 5'-subsegment, provided that said DNA segment is the first DNA segment or fourth DNA segment; or (ii) the 5'-terminus of the

-49-

3'-subsegment, provided that said DNA segment is the second DNA segment or the third DNA segment, provided further that, if both the first and second DNA segments, or both the first and fourth DNA segments, or both the second and third DNA segments, or both the third and fourth DNA segments, consist of a poly(dC) tail or a poly(dG) tail ligated to a terminus as provided above in this Claim, all of said tails on all of said DNA segments which have said tails must be of the same of dC or dG.

10 28. A process according to Claim 27 wherein one segment, selected from the group consisting of said first, second, third and fourth DNA segments, consists of a poly(dC) tail or a poly(dG) tail, provided that said tail is ligated to: (i) the 3'-terminus of the
15 5'-subsegment of said segment, if the segment is the first or the fourth; or (ii) the 5'-terminus of the 3'-subsegment of said segment, if the segment is the second or the third.

20 29. A process for testing a sample for the presence of a target DNA analyte, which comprises a target segment of known sequence, which process comprises:

(A) amplifying said target segment or the complement of said target segment to a concentration
25 which is detectable in a nucleic acid probe hybridization assay, said amplification comprising:

(1) annealing to said target segment
(A) a first DNA segment, which has (i) a 5'-terminal phosphate, and (ii) a 5'-subsegment which terminates with
30 said 5'-terminal phosphate and has the sequence complementary to that of a 5'-subsegment, of at least 5 nucleotides, of said target segment and (B) a second DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and
35 has the sequence complementary to that of a

-50-

3'-subsegment, of at least 5 nucleotides, of said target segment, provided that the sequence of a segment which consists of said 5'-subsegment of said first DNA segment ligated to said 3'-subsegment of said second DNA segment
5 is complementary to the sequence of said target segment;

(2) ligating said annealed first and second segments;

(3) strand-separating said target segment from said ligated first and second segments;

10 (4) annealing to said ligated first and second segments (A) a third DNA segment, which has (i) a 3'-terminal hydroxyl and (ii) a 3'-subsegment which terminates with said 3'-hydroxyl and has the same sequence as said 5'-subsegment, of at least 5
15 nucleotides, of said target segment and (B) a fourth DNA segment, which has (i) a 5'-terminal phosphate and (ii) a 5'-subsegment which terminates with said 5'-phosphate and has the same sequence as said 3'-subsegment, of at least 5 nucleotides, of said target segment;

20 (5) ligating said annealed third and fourth segments; and

(6) to the extent necessary to obtain a concentration of said target segment or its complement, that is detectable in said nucleic acid probe
25 hybridization assay,

(i) strand-separating ligated third and forth DNA segments from ligated first and second DNA segments;

30 (ii) annealing unligated third and fourth DNA segments to ligated first and second DNA segments and unligated first and second DNA segments to ligated third and fourth DNA segments;

(iii) ligating said unligated third and fourth DNA segments annealed in accordance with step
35 (6)(ii) and said unligated first and second DNA segments annealed in accordance with step (6)(ii); and

-51-

(iv) repeating steps (6)(i) to

(6)(iii); and

(B) after said amplification process,
testing said sample by said nucleic acid hybridization
5 probe assay for the presence of said ligated first and
second DNA segments or said ligated third and fourth DNA
segments.

30. A process according to Claim 29 wherein
DNA, which comprises the target segment or the complement
10 thereof to be amplified, is made, prior to the
amplification process, by a process which comprises
reverse transcription of RNA which comprises a segment
with the sequence of said target segment or the sequence
complementary to that of said target segment.

15 31. A process according to Claim 29 wherein the
strand-separations are carried out by heating and the
ligations are catalyzed by E.coli DNA ligase.

32. A process according to Claim 30 wherein
strand-separations are carried out by heating and the
20 ligations are catalyzed by E.coli DNA ligase.

33. A process according to Claim 31 wherein
both said 5'-subsegment of said target segment and said
3'-subsegment of said target segment are at least 8
nucleotides in length.

25 34. A process according to Claim 32 wherein
both said 5'-subsegment of said target segment and said
3'-subsegment of said target segment are at least 8
nucleotides in length.

35. A process according to Claim 33 wherein
30 both said 5'-subsegment of said target segment and said
3'-subsegment of said target segment are 100 or fewer
nucleotides in length.

36. A process according to Claim 34 wherein
both said 5'-subsegment of said target segment and said
35 3'-subsegment of said target segment are 100 or fewer
nucleotides in length.

-52-

37. A process according to Claim 35 wherein said first DNA segment consists of said 5'-subsegment thereof, said second DNA segment consists of said 3'-subsegment thereof, said third DNA segment consists of said 3'-subsegment thereof, and said fourth DNA segment consists of said 5'-subsegment thereof.

38. A process according to Claim 36 wherein said first DNA segment consists of said 5'-subsegment thereof, said second DNA segment consists of said 3'-subsegment thereof, said third DNA segment consists of said 3'-subsegment thereof, and said fourth DNA segment consists of said 5'-subsegment thereof.

39. A process according to Claim 35 wherein at least one of said first, second, third and fourth DNA segments consists of a poly(dC) tail or a poly(dG) tail ligated to: (i) the 3'-terminus of the 5'-subsegment, provided that said DNA segment is the first DNA segment or fourth DNA segment; or (ii) the 5'-terminus of the 3'-subsegment, provided that said DNA segment is the second DNA segment or the third DNA segment, provided further that, if both the first and second DNA segments, or both the first and fourth DNA segments, or both the second and third DNA segments, or both the third and fourth DNA segments, consist of a poly(dC) tail or a poly(dG) tail ligated to a terminus as provided above in this Claim, all of said tails on all of said DNA segments which have said tails must be of the same of dC or dG.

40. A process according to Claim 39 wherein one segment, selected from the group consisting of said first, second, third and fourth DNA segments, consists of a poly(dC) tail or a poly(dG) tail, provided that said tail is ligated to: (i) the 3'-terminus of the 5'-subsegment of said segment, if the segment is the first or the fourth; or (ii) the 5'-terminus of the 3'-subsegment of said segment, if the segment is the second or the third.

-53-

41. A process according to Claim 36 wherein at least one of said first, second, third and fourth DNA segments consists of a poly(dC) tail or a poly(dG) tail ligated to: (i) the 3'-terminus of the 5'-subsegment, provided that said DNA segment is the first DNA segment or fourth DNA segment; or (ii) the 5'-terminus of the 3'-subsegment, provided that said DNA segment is the second DNA segment or the third DNA segment, provided further that, if both the first and second DNA segments, or both the first and fourth DNA segments, or both the second and third DNA segments, or both the third and fourth DNA segments, consist of a poly(dC) tail or a poly(dG) tail ligated to a terminus as provided above in this Claim, all of said tails on all of said DNA segments which have said tails must be of the same of dC or dG.

42. A process according to Claim 41 wherein one segment, selected from the group consisting of said first, second, third and fourth DNA segments, consists of a poly(dC) tail or a poly(dG) tail, provided that said tail is ligated to: (i) the 3'-terminus of the 5'-subsegment of said segment, if the segment is the first or the fourth; or (ii) the 5'-terminus of the 3'-subsegment of said segment, if the segment is the second or the third.

43. A process according to Claim 35 or 36 wherein, if ligated first and second DNA segments are detected, at least one of said first DNA segment and said second DNA segment is labeled with ^{32}P ; wherein, if ligated third and fourth DNA segments are detected, at least one of said third DNA segment and said fourth DNA segment is labeled with ^{32}P ; and wherein said detection comprises detection of the decay of said ^{32}P label of said labeled DNA segment or segments.

-54-

44. A process according to Claim 35 to 36 wherein, if ligated first and second DNA segments are detected, said second DNA segment is labeled through its 5'-terminus with biotin; wherein, if ligated third and
5 fourth DNA segments are detected, said third DNA segment is labeled through its 5'-terminus with biotin; and wherein said detection comprises detection of the biotin of said labeled DNA segment or segments through a
10 reaction catalyzed by an enzyme conjugated to avidin or streptavidin complexed with said biotin label.

45. A process according to Claim 44 wherein said enzyme conjugated to avidin or streptavidin is an alkaline phosphatase.

15

20

25

30

35

1/3

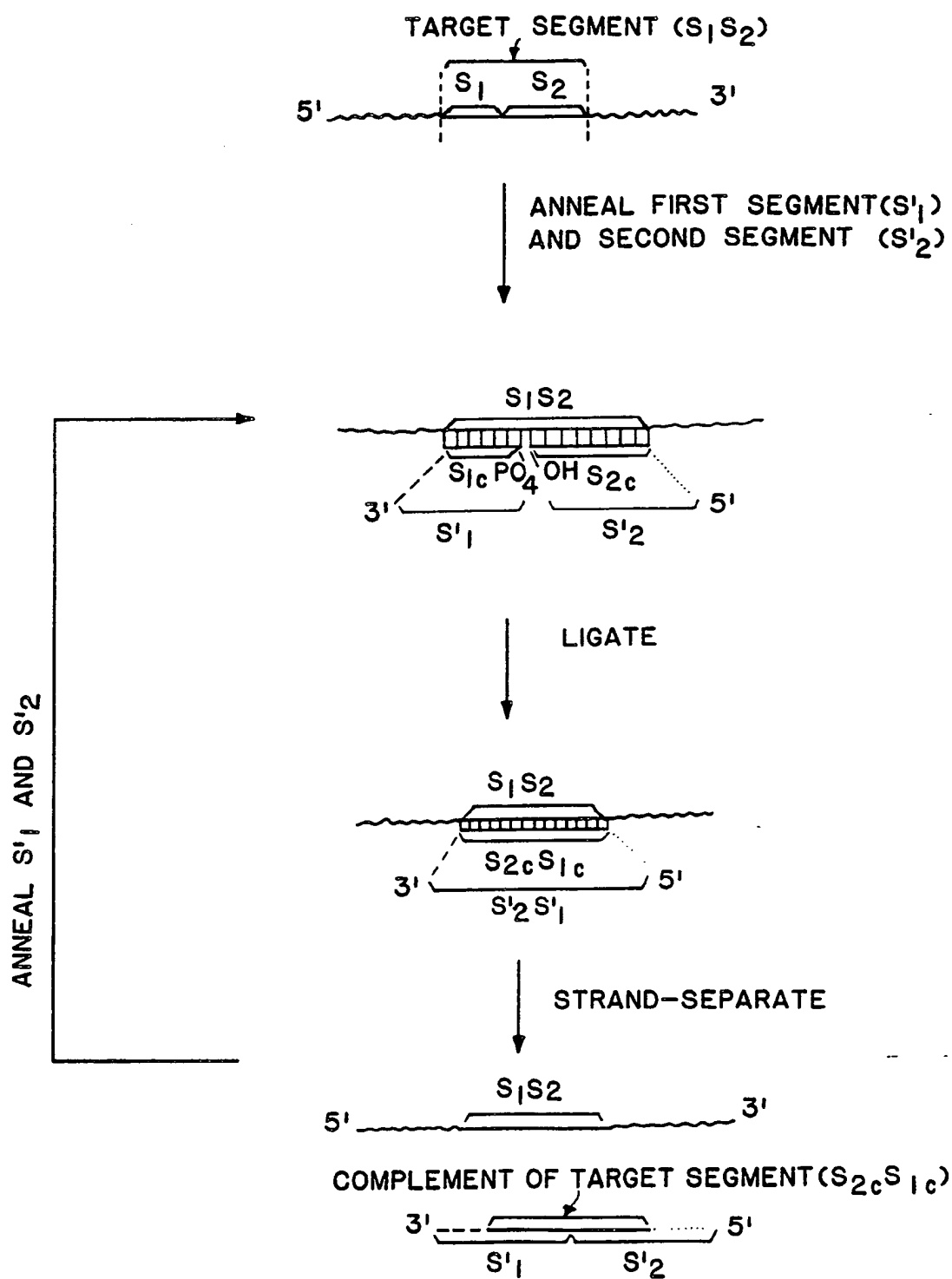
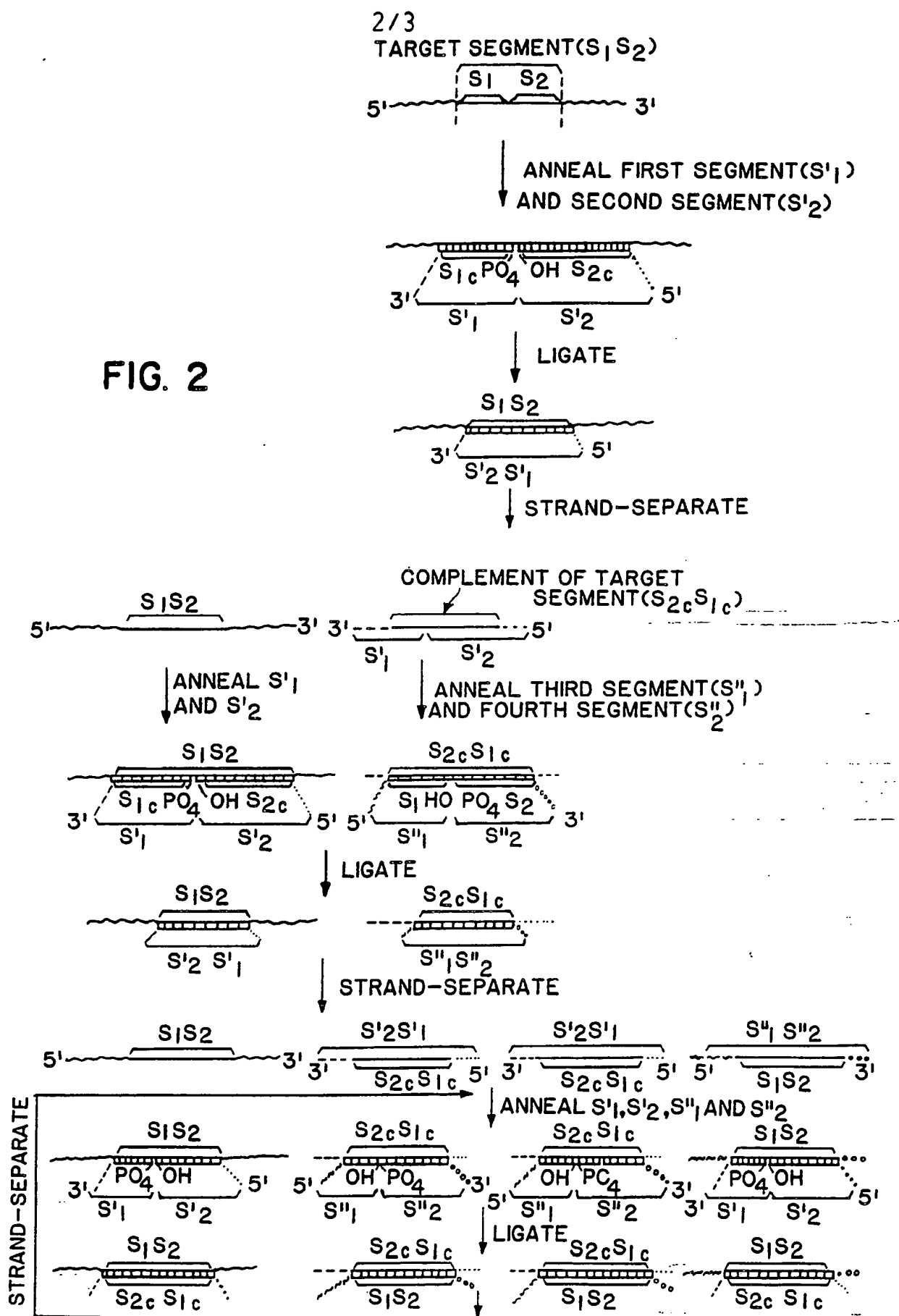


FIG. 1



3/3

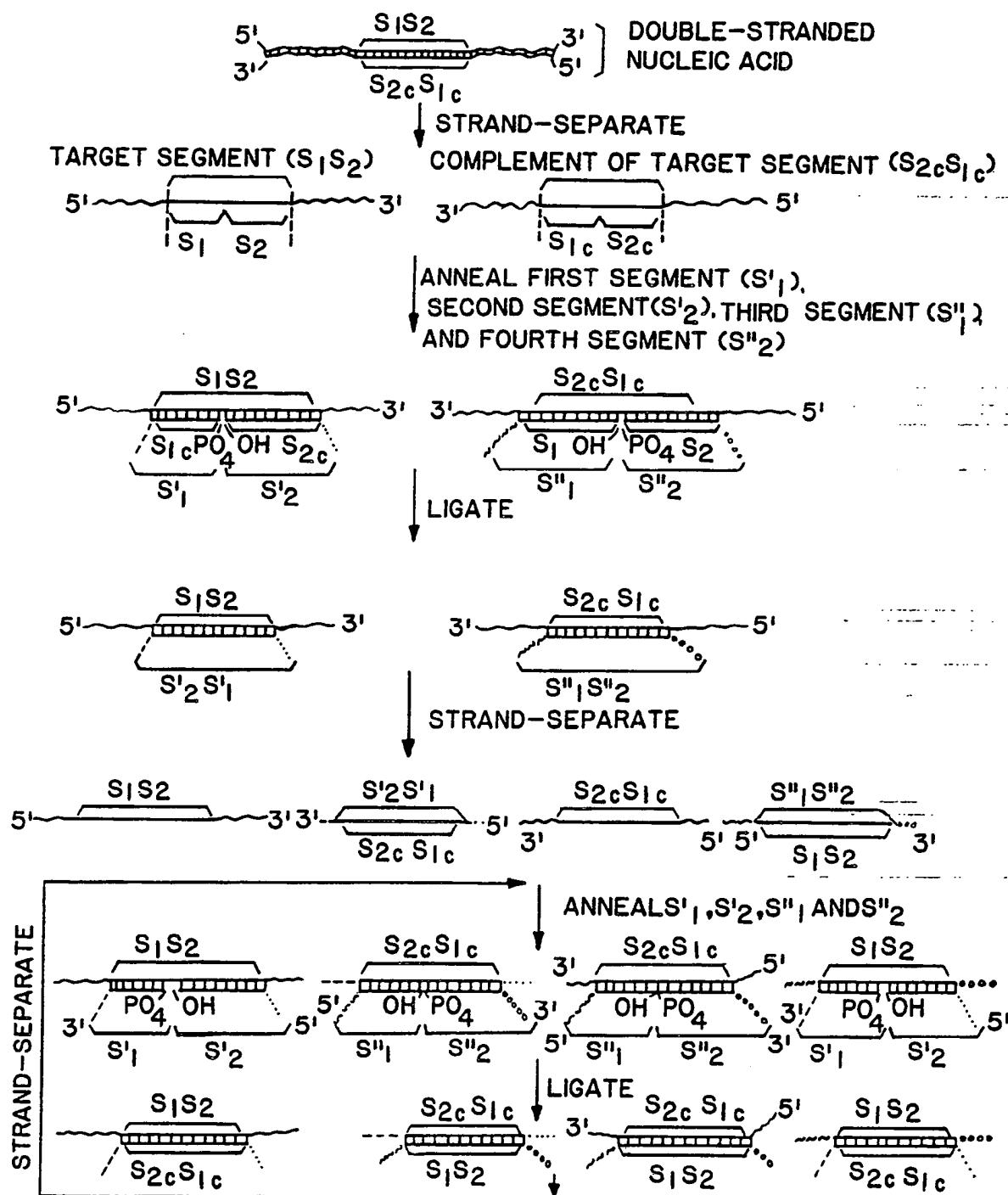


FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/01471**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4) C12Q 1/68; C12P 19/34; C12N 15/00 U.S. 435/6; 435/91; 435/172.3																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;"> 435/6, 91, 172.3, 183, 320 935/8, 16, 78 536/27 </td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <p style="padding: 5px;">Chemical Abstracts Data Base (CAS) 1967-1989; Biological Abstracts Data Base (BIOSIS) 1967-1989. KEYWORDS: LIGASE, AMPLIFY, DNA GENE, COMPLEMENTARY, ANNEAL.</p>			Classification System	Classification Symbols	U.S.	435/6, 91, 172.3, 183, 320 935/8, 16, 78 536/27											
Classification System	Classification Symbols																
U.S.	435/6, 91, 172.3, 183, 320 935/8, 16, 78 536/27																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category *</th> <th style="border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 10%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 4,710,465 (WEISSMAN ET AL) 1 DECEMBER 1987, See entire document, particularly, columns 6, 7, 18 and 19.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-45</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 4,683,195 (MULLIS ET AL) 28 JULY 1987 See entire document, particularly, columns 6-14.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-45</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">US, A, 4,663,283 (KLEID ET AL) 5 MAY 1987 See entire document, particularly, columns 4-6.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-45</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y,P</td> <td style="padding: 5px;">JOURNAL OF MOLECULAR BIOLOGY, Volume 203, No. 2, issued 20 September 1988 (BROWN ET AL) "Functional cDNA libraries from Drosophila embryos", See entire document, particularly pages 425-437.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-45</td> </tr> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	US, A, 4,710,465 (WEISSMAN ET AL) 1 DECEMBER 1987, See entire document, particularly, columns 6, 7, 18 and 19.	1-45	Y	US, A, 4,683,195 (MULLIS ET AL) 28 JULY 1987 See entire document, particularly, columns 6-14.	1-45	Y	US, A, 4,663,283 (KLEID ET AL) 5 MAY 1987 See entire document, particularly, columns 4-6.	1-45	Y,P	JOURNAL OF MOLECULAR BIOLOGY, Volume 203, No. 2, issued 20 September 1988 (BROWN ET AL) "Functional cDNA libraries from Drosophila embryos", See entire document, particularly pages 425-437.	1-45
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³															
Y	US, A, 4,710,465 (WEISSMAN ET AL) 1 DECEMBER 1987, See entire document, particularly, columns 6, 7, 18 and 19.	1-45															
Y	US, A, 4,683,195 (MULLIS ET AL) 28 JULY 1987 See entire document, particularly, columns 6-14.	1-45															
Y	US, A, 4,663,283 (KLEID ET AL) 5 MAY 1987 See entire document, particularly, columns 4-6.	1-45															
Y,P	JOURNAL OF MOLECULAR BIOLOGY, Volume 203, No. 2, issued 20 September 1988 (BROWN ET AL) "Functional cDNA libraries from Drosophila embryos", See entire document, particularly pages 425-437.	1-45															
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; padding: 5px;">12 JULY 1989</td> <td style="text-align: center; padding: 5px;">28 JUL 1989</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding: 5px;">ISA/US</td> <td style="text-align: center; padding: 5px;"> THOMAS D. MAYS </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	12 JULY 1989	28 JUL 1989	International Searching Authority	Signature of Authorized Officer	ISA/US	 THOMAS D. MAYS							
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report																
12 JULY 1989	28 JUL 1989																
International Searching Authority	Signature of Authorized Officer																
ISA/US	 THOMAS D. MAYS																

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y, P

GENE, Volume 71, No. 1, issued August 1988, 1-45
 (KIM ET AL), "Amplification of cloned DNA
 as tandem multimers using BspMI-generated
 asymmetric cohesive ends", See entire doc-
 ument, particularly, pages 1-8.

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ^{1,2} not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed require-
 ments to such an extent that no meaningful international search can be carried out ^{1,2}, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of
 PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
 of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only
 those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to
 the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
 invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.